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Accelerated functional recovery after skeletal muscle ischemia–reperfusion injury using freshly isolated bone marrow cells

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ABSTRACT

Background: Relatively little information exists regarding the usefulness of bone marrow derived cells for skeletal muscle ischemia reperfusion injury (I/R), especially when compared with I/R that occurs in other tissues. The objectives of this study were to eval uate the ability of freshly isolated bone marrow cells to home to injured skeletal muscle and to determine their effects on muscle regeneration.

Materials and methods: Freshly isolated lineage depleted bone marrow cells (Lin BMCs) were injected intravenously 2 d after I/R. Bioluminescent imaging was used to evaluate cell localization for up to 28 d after injury. Muscle function, the percentage of fibers with centrally located nuclei, and the capillary to fiber ratio were evaluated 14 d after delivery of either saline (Saline) or saline containing Lin BMCs (Lin BMCs).

Results: Bioluminescence was higher in the injured leg than the contralateral control leg for up to 7 d after injection ($P < 0.05$) suggestive of cell homing to the injured skeletal muscle. Fourteen days after injury, there was a significant improvement in maximal tetanic torque (40% *versus* 22% deficit; $P < 0.05$), a faster rate of force production (+dP/dt) (123.6 *versus* 94.5 Nmm/S; $P < 0.05$), and a reduction in the percentage of fibers containing centrally located nuclei (40 *versus* 17%; $P < 0.05$), but no change in the capillary to fiber ratio in the Lin BMC as compared with the Saline group.

Conclusions: The homing of freshly isolated BMCs to injured skeletal muscle after I/R is associated with an increase in functional outcomes.

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1. Introduction

Skeletal muscle ischemia–reperfusion injury (I/R) occurs as a result of muscle ischemia followed by the reestablishment of blood flow. Tourniquet application, vascular injury, bone fracture, and skeletal muscle crush injury are among the list of causative factors (reviewed in [1]). There are a large number of options for reducing skeletal muscle I/R if treatments can be applied before, during, or soon after the injury [2–4]. However, in the case of traumatic injuries where I/R is not predictable (e.g., explosions), and an early intervention is desired, such strategies are not as relevant. This is especially the case for severe extremity trauma, wherein lifesaving surgical pro cedures are performed with the objective of stopping hemor rhage for the preservation of vital organ function and the extremities are not the primary focus [5].

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The application of therapies that can be applied and improve regeneration once the patient has been stabilized may be another means to improve outcomes after skeletal muscle I/R. In this regard, there have been a large number of reports describing the successful use of stem cell–based therapies after I/R injury for hepatic [6–8], renal [9–11], and cardiac [12–14] tissues. Consistent with this idea, we have previously demonstrated that muscle progenitor cells (MPCs) enhance tissue repair after skeletal muscle I/R [15]; MPCs were a logical choice given their well documented role in skeletal muscle regeneration; however, the use of MPCs is not currently clinically applicable.

Bone marrow is a rich source of stem and progenitor cells, so it is not surprising that a number of treatments that have progressed to the clinic have used bone marrow cells [13,14,16–18]. Skeletal muscle I/R is a complex injury resulting in vascular, neural, and muscular damage [19], structures that have been shown to benefit from transplanted bone marrow–derived cells in traumatized and diseased conditions [17,20,21]. Recently, it was demonstrated that bone marrow cells (lineage depleted; Lin BMCs) delivered *via* intramuscular injection can survive in a mouse I/R model for up to 1 mo after injury indicating that they may be suitable for the environment imposed with the injury, however, a functional improvement was not realized [22]. Given the ability of systemically delivered stem cells to localize to areas of injury [10,23–25], a logical progression is to evaluate the ability of systemically derived bone marrow cells to home to injured tissue. As the success of regenerative processes are ultimately judged by their capacity to restore function, neural evoked muscle force or torque (which depends on the integrity of neural, vascular, and muscular elements) is an appropriate outcome for which therapies of skeletal muscle I/R injury can be evaluated. To this end, the primary objective of the present study was to determine if bone marrow–derived cells delivered intravenously (IV) would improve functional regeneration after skeletal muscle I/R.

2. Materials and methods

This study has been conducted in compliance with the Animal Welfare Act and the Implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals and was conducted in the animal facility at the US Army Institute of Surgical Research. FVB L2G85 transgenic and FVB mice (The Jackson Laboratory, Bar Harbor, ME) were housed individually in a temperature controlled environment with a 12 hour light–dark cycle.

2.1. Experimental design

To evaluate cell survival and distribution after I/R, Lin BMCs derived from male FVB L2G85 transgenic mice were injected IV into female FVB mice 2 d after I/R. Bioluminescent imaging (BLI) was performed at 0, 1, 3, 7, 14, 21, and 28 d after cell injection (add 2 d for postinjury time) to evaluate cell survival and distribution. To test the hypothesis that BMCs improve regeneration after I/R, 2 d after injury, 15 female FVB mice were allotted to one of two experimental groups: (1) injured with IV injection of saline (Saline, n 8) or injured with IV injection of Lin BMCs derived from male FVB L2G85 transgenic mice (Lin BMCs, n 7). *In vivo* functional assessments were made up to 1 wk before and 16 d after I/R on mice from both experimental groups, after which tissues were collected for histology.

2.2. I/R injury

I/R injury was induced in FVB mice similar to that described previously [26]. Mice were maintained under anesthesia with isoflurane gas (1.5%–2.5%) for the induction of I/R. A pneumatic digit tourniquet (D.E. Hokanson, Inc, Bellevue, WA) was placed as proximal as possible around the elevated upper hind limb of FVB syngeneic mice and inflated to the pressure of 250 mm Hg for a duration of 2 h using a Rapid Cuff Inflator (D.E. Hokanson, Inc). All mice received buprenorphine (0.1 mg/kg, subcutaneously) 30 min before tourniquet release and every 12 h thereafter for the first 24 h.

2.3. Isolation of bone marrow–derived cells

On the day of injection, Lin BMCs were isolated from FVB L2G85 transgenic donor mice (The Jackson Laboratory) for IV delivery to FVB syngeneic recipient mice injured 2 d earlier (The Jackson Laboratory). Only male mice were used as a source of Lin BMCs to minimize potential variability because of the effect of cell gender on regenerative capacity [27]. Magnetic activated cell sorting (MACS) with lineage depletion was used to enrich for stem and progenitor cells, similar to that previously described by us [22] and others [28–33]. Briefly, bone marrow cell suspensions derived from tibia and femurs were labeled with a cocktail of biotin conjugated antibodies against lineage specific antigens (CD5, CD45 R [B220], CD11 b, Gr 1 [Ly 6 G/C], 7–4, and Ter 119) and subsequently magnetically labeled with Anti Biotin MicroBeads (Miltenyi Biotec Inc, Auburn, CA). The lineage positive cells (T cells, B cells, monocytes, macrophages, granulocytes, erythrocytes, and their committed precursors) were depleted by retaining them on a MACS column in the magnetic field of the autoMACS Pro Separator (Miltenyi Biotec Inc, Auburn, CA) while unlabeled lineage negative cells (Lin BMCs) pass through. Lin BMCs from the FVB L2G85 transgenic donors were suspended in saline and injected IV into FVB syngeneic animals injured 2 d earlier to determine (1) their survivability and distribution and (2) the ability to improve muscle function after I/R.

2.4. IV injection

Two days after the induction of I/R mice received either 50 μL of saline only or saline containing 0.2–0.25 $\times$ 10^6 Lin BMCs. Cells were injected through the tail vein using a tuberculin syringe (Tyco Healthcare, Mansfield, MA) and immediately flushed with an additional 50 μL of saline.

2.5. BLI

Cells derived from FVB L2G85 transgenic mice express firefly luciferase allowing for *in vivo* measurements of survival and distribution over time when transplanted into syngeneic FVB recipients [25,34]. Two days after the induction of I/R, FVB

syngeneic mice received either saline or 0.2–0.25 × 10^6 Lin BMCs derived from FVB L2G85 transgenic mice that were isolated on the day of injection. To evaluate cell distribution over time, two animals were evaluated 0, 1, 3, 7, 14, 21, and 28 d after cell delivery. On the day of cell injection animals in the "Lin BMC" group that were tested for functional out comes were also imaged to verify the presence of viable cells. Mice were anesthetized and injected intraperitoneally with D Luciferin (Promega, Madison, WI; 15 mg/kg body weight). Photons emitted from the luciferase expressing cells were collected by a charge coupled device (CCD) camera using a 15 min exposure beginning 5 min after injection. A 1.75 × 0.75 cm region of interest was drawn over the ischemic limb and quantified using Living Image 4.2 software (Caliper Life Sciences Inc, Hopkington, MA). Tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were excised and weighed after the completion of BLI.

2.6. In vivo *muscle function*

Anterior crural muscle (i.e., TA and EDL muscles) isometric torque was measured as a function of stimulation frequency (20–200 Hz) in anesthetized mice (Isoflurane; 1.5%, 1.5 L/min) within 1 wk before and 16 d after I/R injury (14 d after intramuscular injection of Lin BMCs). Only female mice were used for functional analyses to avoid potential effects of gender on functional recovery [35]. Contractile function (i.e., torque–frequency relationship) of the left anterior crural muscles was measured *in vivo* as previously described [36–38]. Anesthetized mice were placed on a heated platform to maintain core body temperature at ~37°C. The left knee was clamped and the left foot was secured to a footplate that was attached to the shaft of a servomotor (300C; Aurora Scientific, Ontario, CA). Both the knee and ankle were positioned at right angles. Sterilized needles were inserted through the skin for the stimulation of the left common peroneal nerve. Stimulation voltage and needle electrode placement were optimized with 5–15 isometric contractions (200 ms train of 0.1 ms pulses at 300 Hz). Contractile function of the anterior crural muscles was assessed by measuring maximal isometric torque as a function of stimulation frequency (20–200 Hz). Core body temperature was monitored with a mouse rectal thermo couple and maintained at ~37°C.

To characterize leftward or rightward shifts in the torque–frequency relationship, individual torque–frequency curves were fit with the following equation:

$$\text{Torque}(x) \quad T_{min} + (T_{max} \quad T_{min}) / \left[1 + (x/\text{Freq}_{50})^{\text{Hill}}\right] \qquad (1)$$

where T_{min} and T_{max} are the smallest (i.e., twitch) and largest (i.e., peak tetanic) respective torques estimated. Freq_{50} is the stimulation frequency at which half the amplitude of force (T_{max}–T_{min}) is reached and Hill is the coefficient describing the slope of the steep portion of the curve.

Measured maximal and minimum torque values were used for statistical comparisons. Rates of torque production (+dP/dt) and relaxation (dP/dt) were calculated from maximal tetanic contractions. TA and EDL muscles were excised and weighed after the completion of functional measurements.

2.7. *Histologic analysis*

After overnight fixation in 4% paraformaldehyde solution at 4°C tissues were soaked in 5% and 20% sucrose in phosphate buffered saline (PBS) for 6 h and overnight, respectively. Tis sues were then frozen in optimal cutting temperature com pound solution in liquid nitrogen, chilled isopentane. Cross sections (10 μM thick) were cut at 20°C and stored at 80° C until staining. The labeling of cell membranes and quantifi cation of centrally located nuclei were done similar to that previously described [19]. Muscle sections were incubated with wheat germ agglutinin Alexa Fluor 594 (Invitrogen, Carlsbad, CA) and mounted using mounting media containing 4′,6 diamidino 2 phenylindole (Invitrogen) to visualize nuclei. Two to three sections (separated by at least 500 μM from three animals per group) per muscle were analyzed to quantify the percentage of fibers containing centrally located nuclei. At least 1000 fibers per muscle in ×200 fields were counted. Capillary to fiber ratio was evaluated in two to three sections (sepa rated by at least 500 μM from three to five animals per group) by staining with Griffonia simplicifolia lectin (Vector Labs, Bur lingame, CA) to identify vessels and tissue autofluorescence used to identify fibers.

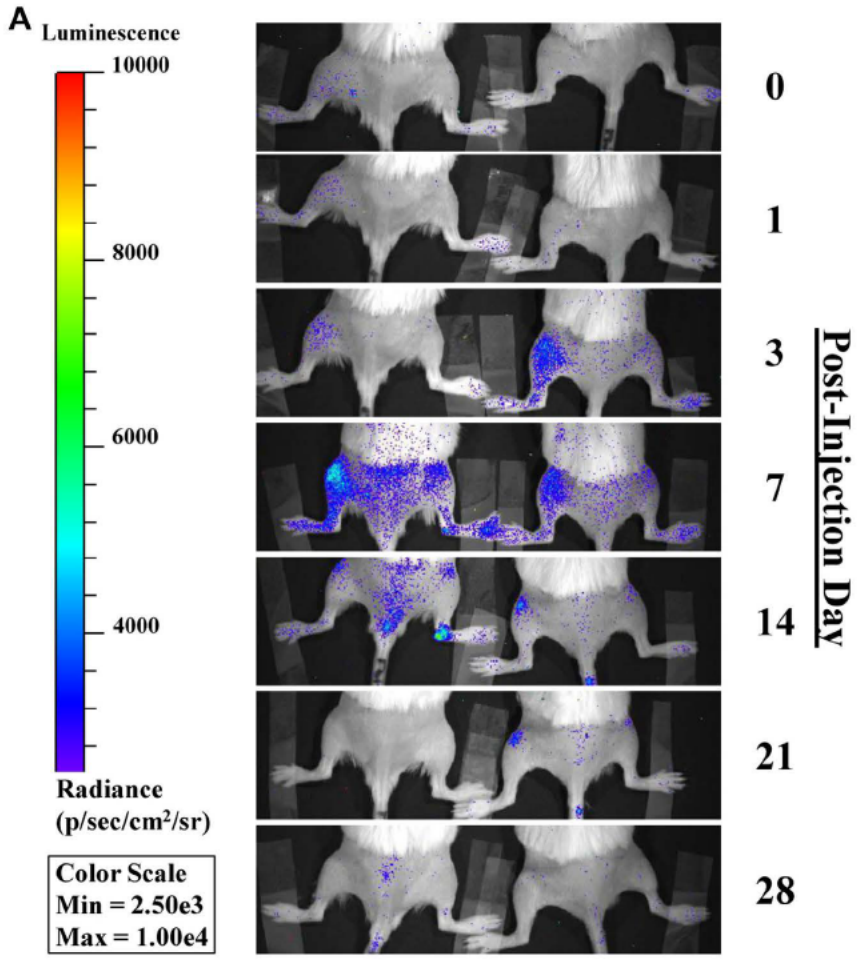
2.8. *Statistical analysis*

Analysis of variance procedures and a t test were used to analyze experimental results using SPSS (Version 18.0, SPSS

Table 1 – Body and muscle weights.

Group	BW (g)		Muscle weight 14 d after injection (mg)			
			TA		EDL	
	Preinjury	Postinjury	INJ	CON	INJ	CON
Saline	19.6 ± 0.5	20.8 ± 0.5	32.0 ± 3.0*	38.0 ± 0.9	6.9 ± 0.6	6.7 ± 0.4
Saline + Lin BMCs	20.7 ± 0.3	21.4 ± 0.2	37.1 ± 2.6*	41.4 ± 1.1	8.0 ± 0.4†	7.8 ± 0.3†

BW = body weight; INJ = injured leg; CON = contralateral control leg.
There was no difference in body weight among any of the groups. All values are mean ± standard error of the mean.
*Main effect for injury (TA).
†main effect for group (EDL).



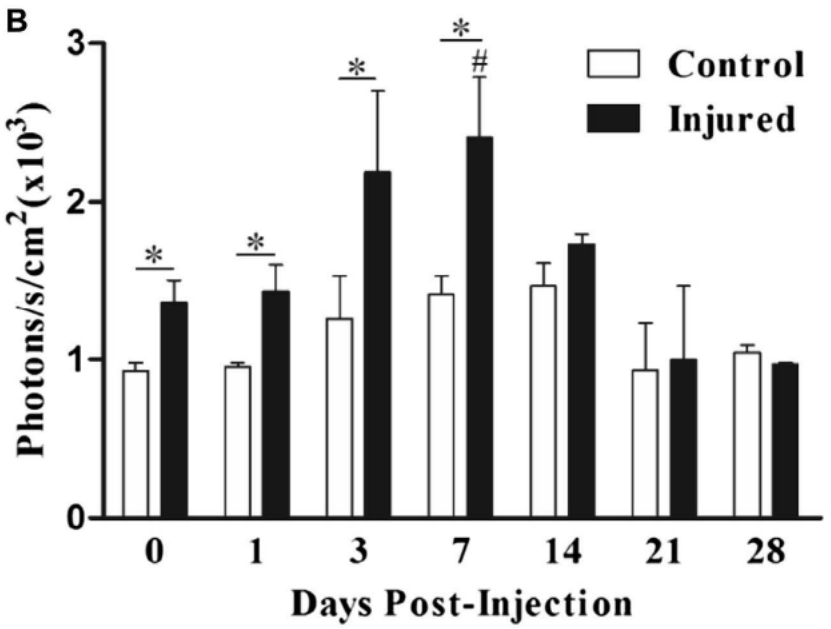


Fig. 1 – BLI was performed 0, 1, 3, 7, 14, 21, and 28 d after IV cell injection to estimate cell homing and survival. Dorsal view images (A) and quantification (B) of BLI after IV injection of cells 2 d after injury. For all images the left leg was the Injured and the right leg was the Control. * Significant difference ($P < 0.05$) between Injured and Control, # Significantly different ($P < 0.05$) from day 0 within Injured. Values are presented as average ± standard error of mean. (Color version of figure is available online.)

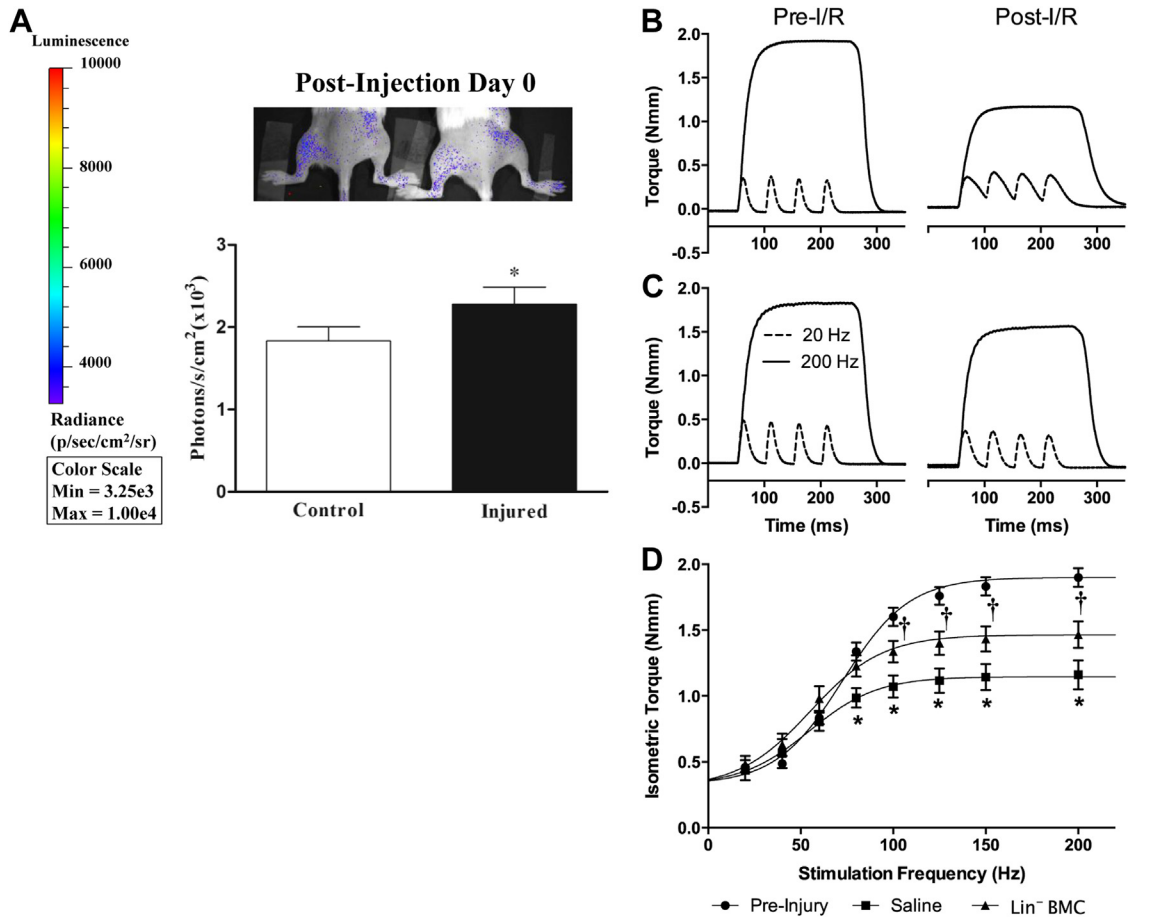


Fig. 2 – Lin⁻ BMCs improve functional recovery after I/R injury. BLI was performed on the day of IV cell injection to verify the presence of viable cells for all animals in the "Lin⁻ BMC" group that were to be tested for functional outcomes. (A) Dorsal view representative images (top right) and quantification (bottom right) of BLI after IV injection of cells 2 d after injury. The left leg was injured and the right leg was the control. Preinjury (left panel) and postinjury (right panel) representative torque responses of anterior crural muscles from saline- (B) and Lin⁻ BMC-treated (C) mice. Note the fusion of contractile response in saline-treated mice at 20 Hz that is not present in the depicted curve for Lin⁻ BMC-treated muscles. Torque–frequency responses (D) measured before and 16 d after injury. Values are mean ± standard error of mean. (A)* Injured ≠ Control ($P < 0.05$), (D)* Saline ≠ Preinjury; † Lin⁻ BMC ≠ Preinjury and Saline ($P < 0.05$). (Color version of figure is available online.)

Inc, Chicago, IL) or SigmaPlot (version 12.0; Systat Software Inc, San Jose, CA) software followed by Fisher's least signifi cant difference (LSD) *posthoc* analyses where appropriate. Differences were considered significant when $P < 0.05$. All values are presented as mean ± standard error of mean.

3. Results

3.1. Body and muscle weights

There were no differences among groups in body weights at the time of functional assessment either before injury or 16 d after injury (Table 1). Injured TA muscle wet weights, regardless of treatment group, were significantly less than their contralateral controls (P 0.023). The EDL muscle wet weights for both the injured and contralateral legs were greater from Lin BMCs than saline injected mice (P 0.020).

3.2. BLI of IV-injected cells

Two days after injury (day 0 of injection, ~30 min after injection) the mean radiance was $1.37 \times 10^3 \pm 0.14$ and $0.92 \times 10^3 \pm 0.05$ p/s/cm^2/sr for the injured and control legs (P 0.048), respectively, indicative of cell homing to the injured leg (Fig. 1). The mean radiance of the injured leg increased above that on day 0 to 2.19×10^3 on day 3 (P 0.060) and 2.41×10^3 on day 7 (P 0.025), suggestive of either continued cell homing to the injured leg or an increase in the proliferation of cells that were present on day 0. By day 14, the decrease in the mean radiance of the injured leg to that comparable of the control leg ($P > 0.05$) persisted throughout the remainder of the evaluation (i.e., through 28 d after injection). The presence of luminescence was also noted in the lower back (presumably the liver, kidneys, and spleen). To verify the presence of viable cells, animals in the Lin BMC group that were tested for functional outcomes (14 d later)

were imaged on the injection day and had a mean radiance of $2.28 \times 10^3 \pm 0.21$ and $1.83 \times 10^3 \pm 0.17$ p/s/cm^2/sr for the injured and control legs ($P < 0.05$), respectively (Fig. 2A).

3.3. In vivo functional outcomes

There was no difference in preinjury isometric torque parameters between groups (Saline *versus* Lin$^-$ BMC: 1.89 ± 0.06 *versus* 1.91 ± 0.12, n 8 *versus* 7; P 0.913) so they were pooled and compared with postinjury torque values for each of the treatment groups. Sixteen days after injury (14 d after saline or Lin$^-$ BMC injection), a 40% loss of maximal tetanic torque was observed in saline injected muscles (Fig. 2; Table 2). In contrast, muscles injected with Lin$^-$ BMCs exhibited a 22% deficit, marking a significant improvement in the functional capacity of muscles after IV Lin$^-$ BMC injection (Fig. 2; Table 2). There were no differences among groups at submaximal stimulation frequencies ranging from 20 to 60 Hz. As a result, the twitch to tetani ratio, calculated as the torque measured at 20 Hz (twitch) divided by maximal torque (tetani), was significantly greater for Saline and Lin$^-$ BMC injected muscles compared with preinjury values (Table 2). In addition, the stimulation frequency at which half the amplitude of torque occurred was shifted similarly to lesser frequencies in both treatment groups after injury (Fig. 2; Table 2). Inspection of the torque waveforms during testing indicated that the functional kinetics of neural elicited torque was altered after injury, and in particular in saline injected muscle (Fig. 2). For instance, in five of six observations, there was a noticeable torque fusion at 20 Hz stimulation that was not observed before injury and was only present in three of seven observations in Lin$^-$ BMC injected muscles. During maximal tetani, the rates of torque production (+dP/dt, $P < 0.05$) and relaxation (−dP/dt) were slower for saline injected muscles after injury; Lin$^-$ BMC injection resulted in a normalization of +dP/dt, but not −dP/dt (Table 2).

Table 2 – *In vivo* functional characteristics.

Measurement	Preinjury	Postinjury (16 d)	
		Saline	Lin$^-$ BMCs
Sample size (n)	15	8	7
Isometric torque (Nmm)			
Tetanic (200 Hz)	1.91 ± 0.06	1.24 ± 0.19*	1.49 ± 0.11*,†
Twitch (20 Hz)	0.42 ± 0.01	0.44 ± 0.07	0.45 ± 0.07
Twitch to tetani	0.24 ± 0.01	0.39 ± 0.09*	0.33 ± 0.06*
+dP/dt (Nmm/s)	141.8 ± 5.3	94.5 ± 10.1*	123.6 ± 11.0†
−dP/dt (Nmm/s)	133.5 ± 6.6	89.8 ± 11.2*	92.1 ± 8.7*
#Freq$_{50}$ (Hz)	75.0 ± 2.2	52.9 ± 5.6*	59.0 ± 4.6*
#Hill coefficient	4.6 ± 0.2	4.1 ± 0.2	4.1 ± 0.4

dP/dt values are from tetanic contractions.
#Parameters were derived from fitting individual force frequency curves with a Hill equation. Group values are mean ± standard error of mean.
* ≠ Preinjury.
† ≠ Saline; $P < 0.05$.

3.4. Histology

There were significantly fewer fibers containing centrally located nuclei in the Saline + Lin$^-$ BMC (17 ± 5%) as compared with Saline only (40 ± 5%), with both experimental groups containing a significantly greater number of fibers with centrally located nuclei than Control (0.4 ± 0.3%; Fig. 3). The capillary to fiber ratio was not different among groups ($P > 0.05$; Fig. 4).

4. Discussion

The therapeutic benefit of cell based therapies, especially those using bone marrow–derived cells (e.g., bone marrow mononuclear cell (MNC) therapies), has been well documented for critical limb ischemia [18], compartment syndrome [17], and a number of applications for myocardial infarction [16]. Experimentally, the use of bone marrow–derived cells, or more specifically, Lin$^-$ BMCs, has been reported to improve outcomes for acute kidney injury [28], diabetic wound healing [29], and myocardial infarction [32,39]; however, their potential therapeutic benefits after skeletal muscle I/R have not been characterized. To address this deficiency, the present study was performed to determine whether the use of Lin$^-$ BMCs is a viable therapy for skeletal muscle I/R. The results from the present study indicate that Lin$^-$ BMCs enhance skeletal muscle tissue regeneration after I/R injury, evidenced by greater functional recovery and lesser tissue damage 16 d after injury (Figs. 2 and 3).

Previously, we observed functional improvements in a rat skeletal muscle I/R model after the intramuscular delivery of culture expanded MPCs despite limited cell survival [15]. In a subsequent study, the intramuscular injection of freshly isolated Lin$^-$ BMCs failed to improve muscle function in a mouse skeletal muscle I/R model despite substantial cell survival [22]. In both cases intramuscular injections were used because we reasoned that the delivery of cells in close proximity to the injured musculature would result in the best outcome. In the present study, we delivered Lin$^-$ BMCs IV to avoid potential damage from intramuscular injections [40] in the mouse and to take advantage of the well documented ability of stem cells to home to injured tissues [7,23,25,33]. Using this approach, Lin$^-$ BMCs were shown to migrate to injured tissue (Figs. 1 and 2), which corresponded to improvements in functional capacity (Fig. 2) and tissue regeneration (Fig. 3) as well as the quality (e.g., dP/dt; Table 2) of injured TA muscles. To the best of our knowledge, this is the first report demonstrating a significant improvement in muscle function after skeletal muscle I/R with the use of bone marrow–derived cells.

It is logical to speculate that although bone marrow is a well recognized source of mesenchymal stem cells (MSCs), and lineage depletion enriches for cells with markers consistent with MSCs [30,31], some of the beneficial effects may be because of the well documented regenerative actions of MSCs. However, extrapolations from cultured MSCs to the putative effects of MSCs within Lin$^-$ BMCs should be made with caution. Bone marrow–derived MSCs that are culture

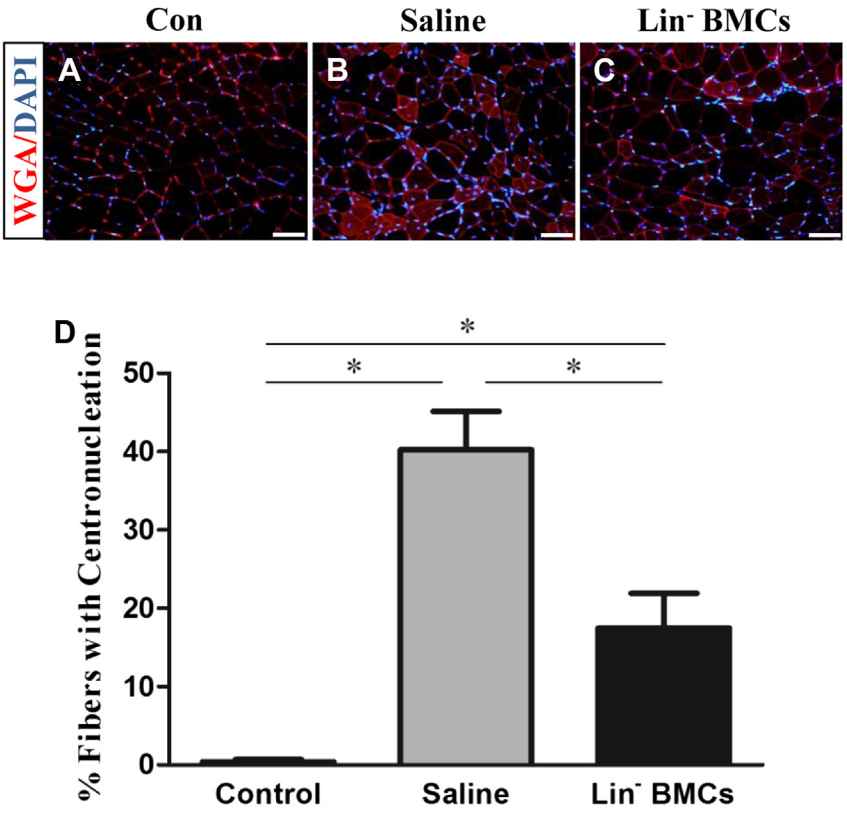


Fig. 3 – Representative images (A–C) and quantification (D) of fibers containing centrally located nuclei 16 d after injury (14 d before injection). Cross sections were stained with wheat germ aggluttin to label cell membranes (red) and counterstained with 4′,6-diamidino-2-phenylindole to identify nuclei (blue). Bar = 50 μM. *Significant difference ($P < 0.05$) between groups. Values are presented as average ± standard error of mean. (Color version of figure is available online.)

expanded are administered in high purity and uncultured MSCs within the Lin fraction of BMCs are in a heterogeneous cellular mixture, effectively diluting the MSC fraction deliv ered. These differences have been shown to manifest in dif ferential angiogenic effects between MSCs and MNCs in critical limb ischemia [41], and to reduce cell migration [42]. Further, the contributions of other stem/progenitor cells that can be found in bone marrow (e.g., endothelial progenitor cells and hematopoietic stem cells) [30,43,44], and the potential effects of delivering multiple cell types simultaneously [45] may all contribute to the regenerative effects of Lin BMCs. Regardless, the Lin BMCs used in this study confer an improvement in therapeutic outcomes after skeletal muscle I/R and reflects the heterogeneity of BMCs (i.e., bone marrow MNCs) currently under investigation clinically [12,13,16–18].

In the present study we do not identify a definitive mech anism by which Lin BMCs, or specific subsets of stem cells contained therein, improved treatment outcomes after I/R injury. There are a number of findings throughout the litera ture that suggest the putative mechanism is likely related to the secretory effects of the stem cells, an idea supported by the observation that conditioned media derived from stem cells is sufficient for improving myocardial viability after I/R [46]. Indeed, there are a large number of paracrine factors secreted by bone marrow–derived cells that have been implicated in improving tissue healing. For example, mesenchymal stem cells have been shown to favorably modulate cytokine production in hepatic [6], renal [9], and cardiac muscle I/R [47–49] models, promoting angiogenesis [50] and proregenerative M_2 macrophage polarization [49,51]. Given the documented angiogenesis promoting effects of stem cells, and the improved functional capacity observed, we reasoned that the therapeutic effect may have been as a result of an improved capillary to fiber ratio, however, this was not observed (Fig. 4). The oxidative stress incurred by skeletal muscle acutely after I/R [52], and the ability of mesenchymal stem cells [6,53,54], bone marrow MNCs [11], and Lin BMCs [33] to suppress inflammation and/or oxidative stress during the acutely after I/R injury [6,11,33,53,54] highlights a potential mechanism whereby BMCs may help to maintain skeletal muscle integrity acutely after skeletal muscle I/R. Thus, it is interesting to consider the possibility that the relatively early delivery of Lin BMCs (2 d after injury) improves functional outcomes by promoting tissue regeneration and/or reducing the damage incurred early in the degenerative phase after I/R.

BLI was used to evaluate cell localization and engraftment of Lin BMCs after IV delivery. It is clear that in addition to homing to the injured leg transplanted cells migrate to other areas (Figs. 1 and 2). This observation is in agreement with others demonstrating that MNCs and purified mesenchymal stem cells may have preference for the liver, bone marrow, spleen, and lungs [24,55]. Nonetheless, within an hour of

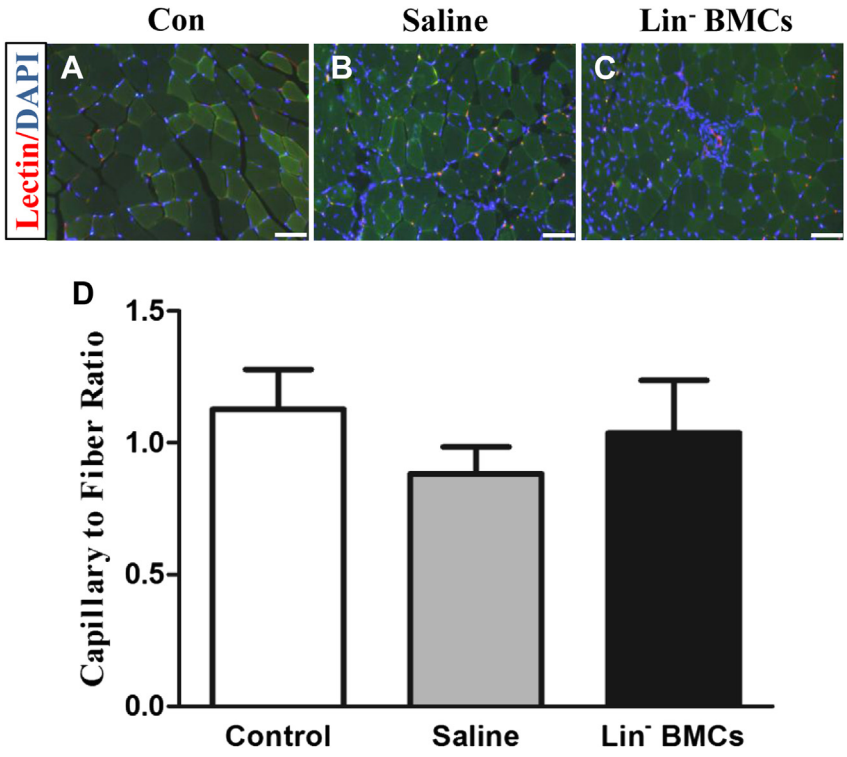


Fig. 4 – Representative images (A–C) and quantification (D) of the capillary-to-fiber ratio 16 d after injury (14 d after injection). Cross sections were stained with Griffonia simplicifolia lectin to label vessels, fibers were visualized with autofluorescence (green), and counterstained with 4′,6-diamidino-2-phenylindole to identify nuclei (blue). Bar = 50 μM. *Significant difference ($P < 0.05$) between groups. Values are presented as average ± standard error of mean. (Color version of figure is available online.)

delivery, cells were found within the injured limb, and the cells persisted there at least a week. Presumably the cells migrated to the areas in the muscle with the greatest need. If stem cell homing follows a chemokine gradient, it is plausible that the areas of greatest damage had the greatest chemokine output and thus the greatest attraction. Although not documented here, the homing of the stem cells to sites of injury may have been limited by the well described entrapment of exogenously delivered cells in clearance organs [24], at least initially. Huang *et al.* [25] described the egress of IV infused endothelial cells derived from embryonic stem cells into ischemic limbs over a period of 14 d. An alternative idea is that although not all of the cells progressed to the injured limb, the presence of cells within the animal was sufficient to improve tissue regeneration without long term engraftment, as has been previously described [56]. Ultimately, the reduction in the percentage of fibers containing centrally located nuclei and the improved functional improvement realized in the present study suggests a therapeutic benefit achieved was regardless of cellular compartmentalization.

The positive findings herein support further exploration of bone marrow–derived therapies for skeletal muscle I/R. The clinical potential of the approach used is supported by the strong foundation supporting the clinical use of cells derived from the bone marrow and of MACs [16–18] and the ability to use a delayed treatment (2 d after injury), which allows time for patient stabilization after traumatic injuries. Importantly, the findings also allude to the possibility that delayed cell based strategies may also have applicability to scheduled microsurgical reconstruction procedures where I/R is predictable. Also, the potential for an autologous approach is supported as this was emulated by using Lin BMCS that were isolated from syngeneic animals and delivered on the same day. Collectively, the data herein support the use of bone marrow as a source of cells for the treatment of skeletal muscle I/R and provide the impetus for further experimentation.

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